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Analysis of the Hepatitis B Virus Precore and ORF-X Sequences in Patients With Antibody to Hepatitis B e Antigen With and Without Normal ALT Levels

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Serum samples from 20 anti-hepatitis B e antigen-positive patients with and without normal alanine aminotransferase (ALT) levels who had serum hepatitis B virus (HBV) DNA detectable only by polymerase chain reation (PCR) were examined. Viral DNA was amplified by PCR, using primers that encompassed precore and ORF-X regions and sequenced directly, to investigate whether mutations in the nucleotide sequences of X and precore gene regions of HBV-DNA might be responsible for the difference in the activity of disease and in the levels of viral replication. The HBV-DNA concentration in patients with abnormal ALT levels was higher than in those with normal ALT. The amount of HBV-DNA correlated with the ALT levels ($P < 0.05$). Seventy-two percent of patients had HBV-DNA harboring the 1896 precore stop mutation, and there was a negative correlation between the percentage of precore mutant genotype and the HBV-DNA concentration ($P < 0.05$). Thirty percent of patients had mutations in ORF-X. Patients with ORF-X mutations had lower levels of HBV-DNA than those who had wild-type virus. The presence of mutations in precore and X regions may be related to a low HBV-DNA concentration and reduced biochemical activity in patients with anti-HBe. ***J. Med. Virol. 56:294–299, 1998.***

KEY WORDS: polymerase chain reaction; HBV-DNA sequencing; mutation; viral replication

INTRODUCTION

Two different phases can be distinguished during chronic hepatitis B virus (HBV) infection: a phase characterized by a high HBV replication level (HBV-DNA detectable by dot-blot hybridization), hepatitis Be antigen (HBeAg) in serum, and high activity of the disease; and a second phase in which HBV-DNA is only detectable by polymerase chain reaction (PCR), anti-Hbe, and normal alanine aminotransferase (ALT) levels [Hoofnagle et al., 1981]. However, there are anti-HBe patients who have elevated ALT levels and serum HBV-DNA detectable by dot-blot hybridization [Bonino et al., 1986]. In these patients, mutations in the precore region of the HBV genome which abolish HBeAg synthesis have been found [Brunetto et al., 1989, 1990; Carman et al., 1989]. Furthermore, mutations in the open reading frame (ORF) X, which encodes for the transcriptional transactivator HBx and contains genetic elements that control viral expression [Kay et al., 1985; Seeger et al., 1986; Siddiqui et al., 1987; Wang et al., 1990; Yun et al., 1992; Zoulim et al., 1994], have been detected in anti-HBe patients with normal ALT levels and low viral replication in comparison with those anti-HBe cases with HBV-DNA detectable by dot-blot hybridization [Fukuda et al., 1995].

However, there is a third class of anti-HBe patients who have active disease (elevated ALT levels) despite the presence of very low HBV replication levels that are only detectable by PCR. In this study, HBV-DNA levels were measured, and the precore region and ORF-X were sequenced from serum samples from 20 anti-HBe patients with and without normal ALT levels who had HBV-DNA only detectable by PCR, to determine the viral factors responsible for these conditions.

MATERIALS AND METHODS

Patients

Serum samples from 20 patients with anti-HBe and with chronic hepatitis B were included in the study. All

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TABLE I. Clinical Data From Patients With Abnormal ALT Levels (Group I) and With Normal ALT Levels (Group II)*

	Group I	Group II
Number of patients	10	10
Mean age (years)[a]	35 ± 10 (20–55)	37 ± 12 (22–59)
Male/female	7/3	5/5
Carrier state (months)[a]	73 ± 57 (15–216)	72 ± 56 (12–150)
Serum ALT (IU/l)[a]	127 ± 110 (49–438)	26 ± 13 (15–45)
Histology		
Minimal CH		
No FB	0	9
Mild FB	3	0
Moderate FB	0	1
Cirrhosis	1	0
Mild CH		
Mild FB	2	0
Moderate FB	1	0
Moderate CH		
Mild FB	1	0
Severe FB	1	0
Cirrhosis	1	0

*CH, chronic hepatitis; FB, fibrosis.
[a]Expressed as mean ± SD (range).

patients had hepatitis B surface antigen (HBsAg) for a mean period of 73 ± 56 months (range, 12–216 months); 10 patients had abnormal serum ALT levels (127 ± 110 IU/l; range, 49–438 IU/l), and the other 10 had normal ALT levels (26 ± 13 IU/l; range, 15–45 IU/l). All patients had liver damage confirmed histologically (Table I).

None of the patients had serum HBV-DNA by dot-blot hybridization, but they were HBV-DNA-positive by PCR in the serum samples studied. None were anti-HCV-, anti-HIV-, or anti-HDV-positive. The patients included in the study had never been treated with antiviral or immunosuppressive therapy. Clinical data of the patients are shown in Table I.

Serologic Markers

HBsAg, HBeAg, anti-HBe, anti-HBs, and anti-HDV were tested by commercial radioimmunoassays (Abbott Laboratories, North Chicago, IL). Anti-HIV 1 and anti-HCV were tested by commercial enzyme-based immunoassays (Abbott Laboratories, and Ortho Diagnostics Systems, Inc., Raritan, NJ). Liver function tests were carried out by standard methods (Smac20, Technicon, New York, NY).

HBV-DNA Quantitation

Although none of the patients had serum HBV-DNA by dot-blot hybridization, the amount of DNA in these samples was quantitated using the Amplicor HBV Monitor test kit, according to the supplier's instructions (Roche Diagnostic Systems, Inc., Basel, Switzerland). Briefly, this assay is based on a single amplification reaction of the target DNA genome of HBV present in the processed sample (50 μl of serum), using one biotinylated and one nonbiotinylated oligonucleotide primer. After amplification, the products were hybridized in parallel in microwells coated with dinitrophenyl (DNP)-labeled oligonucleotide HBV-specific probe and the internal quantitation standard (a synthetic DNA molecule with primer binding sites identical to those of the HBV target and a unique probe sequence specific for this molecule). An anti-DNP-alkaline phosphatase conjugate is used to detect the DNP moiety of the probes. The test quantitates virus titers between 10^3–10^7 viral particles per milliliter of serum. The concentration of HBV-DNA in each sample is calculated from the ratio between the absorbance at 405 nm as detected on a microplate reader for the HBV-specific and for the internal quantitation standard-specific, and converted to HBV-DNA copies/milliliter, using the standard curve prepared in each assay.

HBV-DNA Amplification and Direct Sequencing

Viral DNA was extracted and amplified by PCR, as previously described [Cabrerizo et al., 1996]. The outer primers used for PCR of the ORF-X were 5′-CTTTTGGGCTTTGCTGCTCC-3′ at nt position 1006–1025, and 5′-TTGCCTTCTGACTTCTTTCC-3′ at nt position 1955–1974; for nested PCR, the primers used that encompassed the ORF-X and the sequences were 5′-CAATTCTGTCGTCCTCTCG-3′ at nt position 1335–1353, and 5′-CCTCCAAGCTGTGCCTTG-3′ at nt position 1869–1886 [Ono et al., 1983].

Target primers used for PCR of the precore region were previously reported [Cabrerizo et al., 1996].

In order to avoid false-positive results, the contamination prevention measures described by Kwok and Higuchi [1989] were followed. Appropriate negative controls were included in each PCR assay, and each sample was tested twice by different workers in independent experiments; in all cases, 100% concordance was obtained.

Amplified DNA was purified with the Qiaquick PCR purification kit (Qiagen GmbH, Hilden, Germany), and Cy5 direct sequencing of the ORF-X region products was performed using the ALF™ Express DNA Automated Sequencer (Pharmacia Biotech AB, Uppsala, Sweden).

The predominant HBV precore variant, with a single change from G to A at position 1896, was determined in the serum samples by a specific oligonucleotide hybridization assay of nested PCR products, using probes for the wild-type HBV and precore mutant at nucleotide position 1896, as described previously [López-Alcorocho et al., 1994].

Statistical Analysis

Data were expressed as mean ± SD and examined using Student's t-test and Pearson's correlation coefficient.

RESULTS

HBV-DNA Quantitation

Serum HBV-DNA was quantified using the Amplicor HBV Monitor test. The mean HBV-DNA levels in the

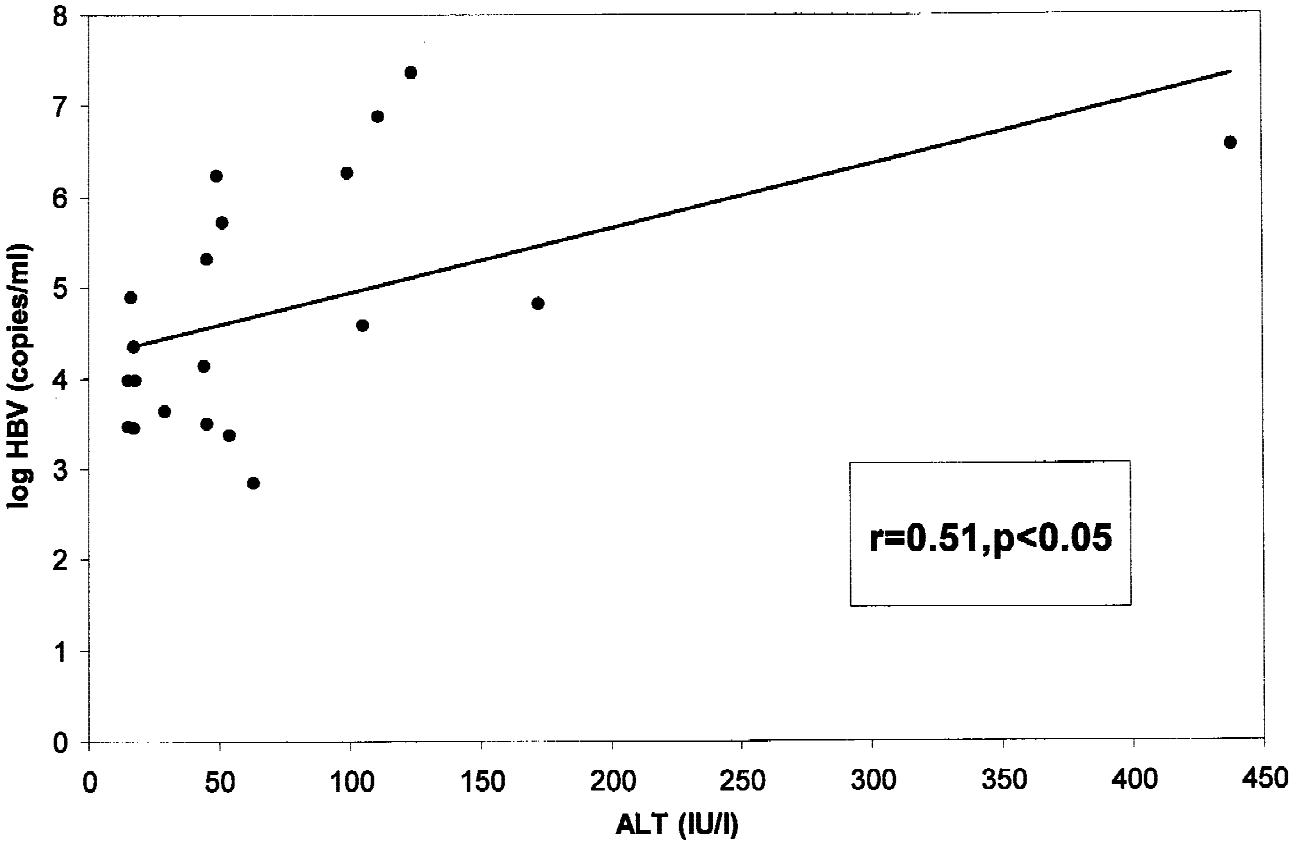

Fig. 1. Correlation between serum ALT levels and HBV-DNA concentration, when all patients were considered together (Pearson's correlation coefficient).

serum samples of patients with abnormal ALT levels ($3.2 \pm 6.8 \times 10^6$ HBV copies/ml) were higher than in those of patients with normal ALT levels ($3.5 \pm 6.1 \times 10^4$ HBV copies/ml), but the difference did not reach statistical significance.

There was a significant positive correlation between serum HBV-DNA concentration and ALT levels ($r = 0.51$, $P < 0.05$) when all patients were considered together (Fig. 1).

ORF-X Mutations in the HBV Genome

Regarding the HBV sequences isolated from the serum of patients with abnormal ALT levels, the ORF-X sequence corresponded to the wild-type HBV genome in 8 cases. The HBV ORF-X sequence from the remaining 2 patients displayed nucleotide substitutions with respect to the published HBV-DNA sequences [Ono et al., 1983]. In one patient, G to A substitution at ORF-X nucleotide position 200 (glycine to aspartic acid change at HBx amino-acid position 67) was detected, and in the other patient, G to A substitution at ORF-X nucleotide position 407 located in the core promoter (glycine to aspartic acid at HBx amino-acid position 136), and C to A change at nucleotide position 461, which was situated in the DR II region (alanine 154 was substituted by aspartic acid), were found.

With respect to the patients with normal ALT levels, the ORF-X sequence in the serum samples from 6 patients was identical with the prototype sequence, whereas in 4 patients nucleotide substitutions in ORF-X were found; G to T change at nucleotide position 169 (glycine to cysteine at amino-acid position 57) of ORF-X from 2 patients, G to A and T to G substitutions at nucleotide positions 169 and 183, respectively (glycine to serine at amino-acid position 57 and cysteine to tryptophan at position 61, respectively) in another, and a T insertion at nucleotide position 7, which generates a stop codon in the remaining patient, were detected (Table II).

The HBV-DNA level in the 6 patients with mutations in the ORF-X region was lower ($3.2 \pm 6.7 \times 10^5$ HBV copies/ml) than in the patients who had the wild-type sequence ($2.6 \pm 6.1 \times 10^6$ HBV copies/ml), although the difference was not statistically significant. When each group of patients (with or without normal ALT levels) was considered separately, the patients with ORF-X mutations also had lower levels of HBV-DNA than those with a wild-type genotype ($2.9 \pm 3.0 \times 10^4$ vs. $4.0 \pm 7.4 \times 10^4$ HBV copies/ml, respectively, in patients with normal ALT levels, and $9.1 \pm 9.1 \times 10^5$ vs. $4.6 \pm 7.4 \times 10^6$ HBV copies/ml in patients with abnormal ALT levels).

TABLE II. HBV-DNA Concentration and Sequencing Results of ORF-X and Distribution of Precore Mutants in Patients With Abnormal (Group I) and Normal (Group II) ALT Levels

	HBV copy number/ml	Precore region sequence	X-gene region sequence
Group I			
1	2.36×10^3	Mixture (18/82)[a]	Wild type
2	1.83×10^6	Mixture (71/29)	Gly67Asp
3	7.01×10^2	Mixture (67/33)	Gly136Asp/Ala154Asp
4	3.56×10^6	Mixture (83/17)	Wild type
5	7.53×10^6	Wild type	Wild type
6	1.69×10^6	Wild type	Wild type
7	6.45×10^4	Mutant 1896	Wild type
8	5.14×10^5	Wild type	Wild type
9	2.33×10^7	N.D.	Wild type
10	3.78×10^4	N.D.	Wild type
Group II			
1	2.88×10^3	Mixture (59/41)	Wild type
2	1.35×10^4	Mixture (56/44)	Wild type
3	7.94×10^4	Mixture (35/65)	T-insertion: stop
4	4.30×10^3	Mixture (5/95)	Wild type
5	3.10×10^3	Wild type	Gly57Ser/Cys61Trp
6	2.80×10^3	Mixture (2/98)	Wild type
7	2.24×10^4	Mixture (37/63)	Gly57Cys
8	9.62×10^3	Mixture (16/48)	Gly57Cys
9	2.06×10^5	Wild type	Wild type
10	9.35×10^3	Mutant 1896	Wild type

[a]Mixture (percent wild-type/percent mutant 1896). N.D., not done.

Precore Region Mutations in the HBV Genome

In the serum sample from patients with normal ALT levels, 2 (20%) had wild-type, one (10%) had only the mutant 1896 genotype, and the remaining 7 (70%) had a mixture of wild-type and precore mutant. Of the patients with abnormal ALT levels, 3 (37%) had a wild-type HBV genome, only one (13%) had the precore mutant at nucleotide 1896, 4 (50%) had a mixture of genotypes, and in 2 cases, no serum was available for HBV genotyping (Table II).

Patients with precore mutations had lower HBV-DNA levels than the patients with only the wild-type sequence ($4.3 \pm 0.9 \times 10^5$ vs. $1.9 \pm 2.8 \times 10^6$ HBV copies/ml), but the difference was not statistically significant. Also, in patients with normal ALT levels, those with the precore mutation had lower HBV-DNA ($1.8 \pm 2.4 \times 10^4$ HBV copies/ml) than those with the wild-type sequence ($1.1 \pm 1.0 \times 10^5$ HBV copies/ml); this finding was also obtained in patients with abnormal ALT values: those with precore mutants, $1.1 \pm 1.4 \times 10^6$ HBV copies/ml vs. those with wild-type genome, $3.2 \pm 3.1 \times 10^6$ HBV copies/ml.

There was a significantly negative correlation between HBV-DNA concentration and the percentage of precore mutant genotype ($r = -0.54$, $P < 0.05$) (Fig. 2) when all patients were considered, but no correlation between ALT and precore mutants was found (data not shown).

DISCUSSION

HBV mutants which have mutations in the precore region and in ORF-X have been reported [Carman, 1995; Repp et al., 1992; Uchida et al., 1995], but it is not clear whether such mutations may be the cause of the different levels of viral replication and ALT in patients with anti-HBe.

HBV-DNA levels were measured in serum samples from 20 anti-HBe patients, with and without abnormal ALT levels. The concentration of serum HBV-DNA was determined using the Amplicor HBV-DNA Monitor test. This is the first time that a kit based on PCR and hybridization has been used to quantitate serum HBV-DNA in patients without HBV-DNA detectable by dot-blot hybridization. The test uses a series of standard amounts of HBV-DNA to measure virus titers between 10^3–10^7 viral copies/ml of serum. Our results are within this range.

HBV-DNA levels in patients with abnormal ALT values were higher than those with normal ALT levels, although the difference did not reach statistical significance. When all patients were considered together, the HBV-DNA level correlated with the ALT levels ($P < 0.05$). These findings show that the biochemical activity of the disease is related directly to the HBV-DNA levels in this group of patients. Apart from immune-mediated cytolysis of the infected hepatocytes, the results suggest that HBV seems to be able to damage directly the hepatocytes in these patients.

On the other hand, 72% of the patients included in this study were infected by HBV variants harboring the precore mutation at the 1896 nucleotide position, either alone or together with wild-type HBV. The prevalence of precore mutants was similar in patients with or without normal ALT (80% and 62%, respectively), and the presence of the precore mutants by itself does not explain the differences in ALT levels between both group of patients [Tur-Kaspa et al., 1992]. However, a negative correlation was observed between

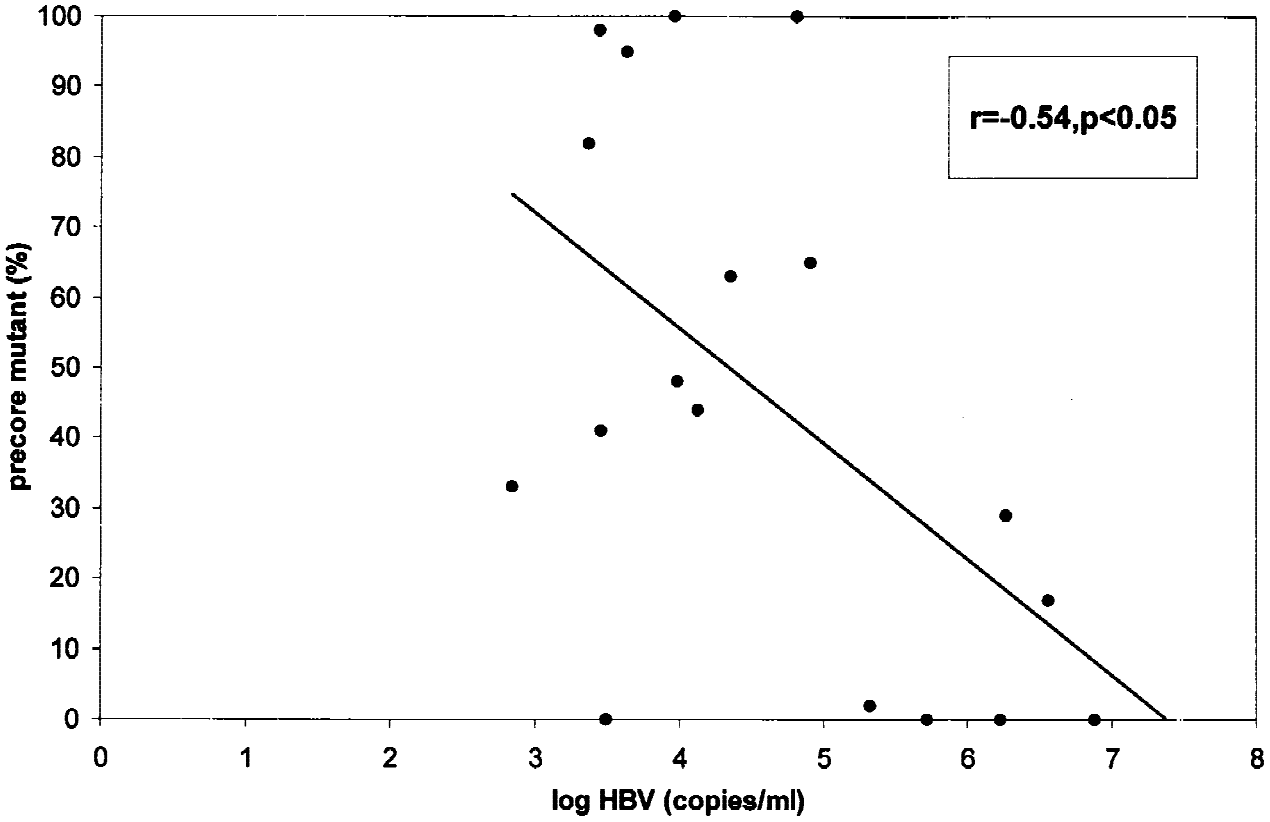

Fig. 2. Correlation between serum HBV-DNA concentration and percentage of the precore mutant genotype in each patient (Pearson's correlation coefficient).

the percentage of HBV particles having the precore mutant genotype in each patient and the concentration of HBV-DNA ($P < 0.05$). Therefore, lower levels of HBV-DNA corresponded to higher percentages of precore mutant HBV genomes. This finding agrees with that reported in ducks infected with the duck hepatitis B virus (DHBV) [Chuang et al., 1994], who showed a lower replication level of DHBV with a stop codon in the precore region of the viral genome, in comparison with the wild-type. Thus, the precore mutation at nucleotide 1896 was located in the encapsidation signal region of precore mRNA, and it might interfere with virus replication, especially in competition with the wild-type virus. The HBe-minus mutation at nucleotide 1896 disrupts a base pair of the stem-loop structure of the encapsidation signal, and may diminish the RNA packaging [Tong et al., 1993]. An additional nucleotide change might restore base pairing by sequence covariation, but in our patients, due to the hybridization technique used to detect precore mutants, it is unknown whether this second mutation was present or not. On the other hand, although most of the patients studied were infected by wild-type HBV alone or together with the precore mutant, HBeAg was undetectable in all patients. Taking into account the low viral replication level in these patients, the lack of HBeAg detection may have been due to the fact that HBeAg is being synthesized at a very low level, undetectable by conventional techniques.

Mutations were observed in the ORF-X sequences in only 30% of the total of the anti-HBe patients studied. Furthermore, the frequency of ORF-X mutations was similar in HBsAg carriers with normal or abnormal ALT levels (40% and 20%, respectively). These results differ from a previous report [Fukuda et al., 1995], since in that study, 17/19 asymptomatic carriers had ORF-X mutations but none of the 9 symptomatic patients carried the mutations. This difference may be due to the type of symptomatic patients included, since none of our patients had serum HBV-DNA by dot-blot hybridization, while all the symptomatic patients from Fukuda et al. [1995] did. In addition, differences in the prevalence of HBV genotypes between Spain and Japan may explain the discrepancy [Tachinaba et al., 1989; Wallace et al., 1994]. Furthermore, the ORF-X mutations described in other studies [Fukuda et al., 1995; Moriyama, 1997; Uchida et al., 1995] consisted in point or 8–20-bp deletions that resulted in the truncation of the X protein, while the mutations found in our patients were point nucleotide substitutions, which generate changes in the amino-acid sequence and may have less biological significance than the others. Patients infected by HBV with mutations in ORF-X had lower levels of viral DNA than those infected with the

wild-type virus in this region. This finding suggests that ORF-X mutations alter the transactivating activity of HBx. However, this requires functional analyses of these mutants.

Thus, with respect to ORF-X, there are three different situations: symptomatic patients who have high serum HBV-DNA levels (detectable by dot-blot) and without ORF-X mutations; asymptomatic carriers (normal ALT levels) with mutations in ORF-X that produce very low levels of viral replication [Fukuda et al., 1995]; and anti-HBe patients with abnormal ALT levels but with low serum HBV-DNA concentration (only detectable by PCR), who have mutations in ORF-X that might be the cause of the decrease in viral replication levels.

Finally, another important aspect is the relative infectivity of the patients. First, these patients have a very low levels of circulating HBV particles and therefore, their infectivity should be low. Second, the mutations in ORF-X in in vitro studies had demonstrated that the X-gene product is not necessary for the production of HBV particles in transfected cells [Blum et al., 1992]; by contrast, in vivo studies in woodchucks infected with the woodchuck hepatitis virus (WHV) lacking a functional ORF-X suggest that it is important for the establishment of chronic infection [Chen et al., 1993]. Thus, these findings support the notion that the infectivity of these patients must be low.

In conclusion, our results suggest that lower levels of viral replication correspond with lower biochemical activity of the disease, and that they are related to the presence of mutations in ORF-X and/or in the precore region in patients with anti-HBe.

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